Sabouraudia (1977), **15,** 79–85

IDENTIFICATION AND PURIFICATION OF THE SPECIFIC ANTIGEN OF *PARACOCCIDIOIDES BRASILIENSIS* RESPONSIBLE FOR IMMUNOELECTROPHORETIC BAND E

L. A. YARZABAL*, D. BOUT*, FRIDA NAQUIRA*, JEANINE FRUIT* AND SUZANNE ANDRIEU**.

Service d'Immunologie et de Biologie Parasitaire (Directeur: Professeur A. Capron), Faculté de Médecine et Institut Pasteur, Lille,* 59000, *France; and* *Unité INSERM U42 de Biologie et d'Immunologie Parasitaires et Fongiques (Directeur: Professeur J. Biguet) Villeneuve D'Ascq,* 59650, *France*

A new purified antigen (E_2) of *Paracoccidioides brasiliensis* mycelial growth phase was isolated by immunoadsorption from a crude metabolic soluble extract of the fungus. The antiserum prepared in a rabbit by inoculation of E_2 antigen developed only one immunodiffusion line with the crude metabolic extract. Findings on immunological analysis showed that E_2 antigen is the antigenic component of immunoelectrophoretic band E. The isolated antigens did not possess detectable alkaline phosphatase activity. It reacted in immunodiffusion tests with all the sera (14/14) from *P. brasiliensis* infected patients containing precipitating antibodies.

Recently, we described an immunoadsorption procedure which permits the purification and isolation of soluble antigens of *Paracoccidioides brasiliensis* (9). During the course of the research a rabbit was immunized with the immunocomplex corresponding to the immunoelectrophoretic band E, characteristic of patients with evolutive paracoccidioidomycosis (7). The resultant anti-serum contained antibodies against two of the soluble antigens from crude metabolic extract of the *P. brasiliensis* mycelial phase. One of these antigens, designated E_1, was isolated and partially characterized in the anterior work (9). It was shown to have a cationic electrophoretic mobility and alkaline phosphatase activity. The other, designated E_2, has now been purified employing the same methodology.

In this article, we describe the procedure for the purification of antigen E_2, give evidence which shows that this antigen is responsible for band E formation, and present preliminary results concerning its serological reactivity to human antisera.

Materials and Methods

Antigens

Crude metabolic extracts of *P. brasiliensis* (IHM† 1572 strain), *Aspergillus fumigatus* (strain supplied by J. L. Longbottom), *Blastomyces dermatitidis* (strain supplied by E. D. McDonough), *Histoplasma capsulatum* (IHM 1524 strain) were prepared. The procedure used was the same described in our earlier work (9). All strains are stocked in the INSERM U42, Lille, France.

Experimental antisera

Following the methodology detailed in the preceding article (9), the following antisera were prepared in white rabbits weighing 3 kg each: (i) anti-*P. brasiliensis*

† IHM = Instituto de Higiene de Montevideo, URUGUAY.



crude metabolic extract (RIS anti-Pb), (ii) anti-band E (RIS anti-band E), (iii) anti-band EI (MSRIS I), and (iv) anti-band EII (MSRIS II). The bands EI and EII were identified on an immunoelectrophoregram, excised and injected separately into two rabbits according to (6). Antisera to crude metabolic extracts of *A. fumigatus*, *B. dermatitidis* (RIS anti-Bd) and *H. capsulatum* (RIS anti-Hc) were also raised in rabbits according to the protocol detailed in our previous paper (9). The monospecificity of MSRIS I and MSRIS II was determined by means of double diffusion and immunoelectrophoretic technics, against crude metabolic extracts of *P. brasiliensis* and the heterologous antigens.

Human sera

Fourteen sera from confirmed cases of paracoccidioidomycosis and two sera from confirmed cases of histoplasmosis were utilized. Ten of the paracoccidioidomycotic sera were taken from Colombian patients studied by Dr. Angela Restrepo (Depto. de Microbiologia y Parasitologia, Facultad de Medicina, Universidad de Antioquia, Medellin, Colombia). The other four and two histoplasmosis sera were taken from Venezuelan patients diagnosed by Dr. Maria B. de Albornoz (Seccion de Micologia, Instituto de Dermatologia, Caracas, Venezuela).

Purification of E_2 antigen

The antigen was isolated from the crude metabolic extract of *P. brasiliensis* by means of affinity chromatography. In the first step the immunoglobulins of the monospecific anti-E_2 antigen antiserum were fixed to CNBr activated Sepharose 4B (Pharmacia, Uppsala, Sweden) using the method of Axen, Porath and Ernback (1). In a second step the crude extract was chromatographed through a column charged with the immunoadsorbent. Finally, the antigen retained in the column by the antibodies was eluted in 0·2 M glycine-HCl, 0·5 M NaCl, pH 2·8 buffer.

Immunochemical analysis

The immunological study was done by means of immunodiffusion and immunoelectrophoretic techniques testing the final product against the antisera to crude homologous and heterologous antigens. To test the purity of the final product a rabbit was injected intradermally, as described by Vaitukaitis *et al.* (6), with approximately 500 μg of the purified antigen. Its enzymatic activity was investigated according to the methods described by Uriel (5). The enzymes sought were: α-esterase, β-esterase, glucose-6-phosphate dehydrogenase, l-alanine dehydrogenase, lactic dehydrogenase, malic dehydrogenase, and alkaline phosphatase.

Serological reactivity

All human sera were tested simultaneously, in the same double diffusion slide, against the crude metabolic extract of *P. brasiliensis* and the purified E_2 antigen. Each serum was previously analysed by immunoelectrophoresis against crude metabolic extracts of *P. brasiliensis* and *H. capsulatum*. The results were interpreted in all cases after searching for alkaline phosphatase activity and staining with amido Schwartz.



Results

Production of the monospecific anti-E_2 antiserum

The antiserum resulting from the immunization of a rabbit with band EII revealed only one of the antigenic components of the crude metabolic extract of *P. brasiliensis* in the immunodiffusion tests (fig. 1). It did not react with the crude heterologous antigens.

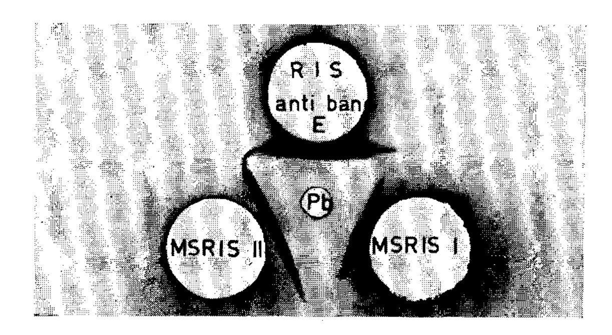


Figure 1.—Double diffusion analysis of *P. brasiliensis* metabolic extract (Pb) by rabbit antisera anti-band E (RIS antiband E), anti-band EI (MSRIS I) and anti-band EII (MSRIS II).

Isolation of E_2 antigen

The product eluted from the column of immunoadsorbent was nonpigmented. The yield was small: 60 mg of crude extract gave about 800 μg of purified antigen, calculated from the optical density in a Beckman Spectrophotometer DB-T at 280 nm, with bovine albumin as reference protein. The immunological analysis of E_2 antigen by RIS anti-Pb in immunoprecipitation tests, produced only one precipitation line (figs. 2 and 3) while the heterologous antisera did not react with the purified antigen (fig. 2).

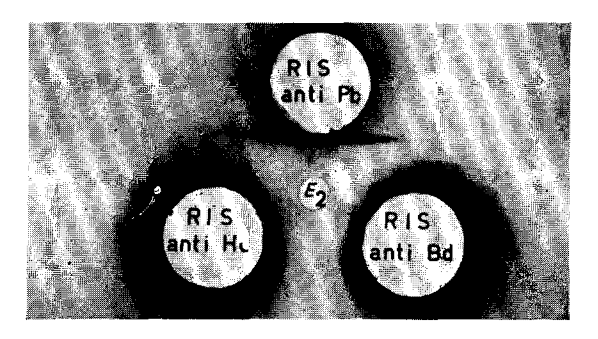


Figure 2.—Double diffusion analysis of E_2 antigen by immune sera to crude metabolic extract of *P. brasiliensis* (RIS anti-Pb), *B. dermatitidis* (RIS anti-Bd) and *H. capsulatum* (RIS anti-Hc).



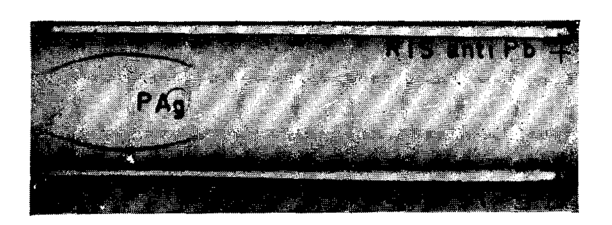


Figure 3.—Immunoelectrophoretic analysis of E_2 antigen (Pag) by antiserum to crude metabolic extract of *P. brasiliensis* (RIS anti-Pb) and antiserum to band EII (MSRIS II).

Immunochemical characterization of antigen E_2

The rabbit inoculated with the product eluted from the immunoadsorbent column produced a monospecific antiserum against antigen E_2 (fig. 4). The precipitating system formed by the purified product and the rabbit anti-crude metabolic extract of *P. brasiliensis* antiserum is localized in the cationic area of the immunoelectrophoregrams. This system does not possess any of the enzymatic activities under consideration in the present work. The precipitation band formed by the monospecific antiserum anti-E_2 with the crude extract of *P. brasiliensis* gave origin to a reaction of total immunological identity with that produced by the same antigen by a patient serum which only forms the band E during immunoelectrophoresis (fig. 5).



Figure 4.—Immunoelectrophoretic analysis of metabolic extract of *P. brasiliensis* by antisera to band E (RIS anti-band E) and anti-E_2 antigen (RIS anti-E_2).

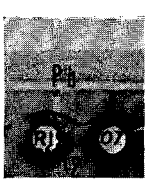


Figure 5.—Identity reaction between antiserum to E_2 antigen (RIS anti-E_2) and the serum of a patient containing only the antibody responsible for band E (07).

Primary evaluation of serological reactivity of E_2 antigens

When testing the purified antigen and the crude metabolic extract of *P. brasiliensis* by double diffusion with the sera from the 14 patients with proved paracoccidioidomycosis and the 2 infected by *H. capsulatum* both antigens revealed the same sensitivity (Table 1), but the use of the purified antigen eliminated the cross-reaction of the



2 histoplasmosis sera. The serum 07 (see Table 1), which formed only one band in immunoelectrophoresis against the crude extract, reacted also with the purified antigen in double diffusion.

TABLE 1.—COMPARED SEROLOGICAL REACTIVITY OF A CRUDE METABOLIC EXTRACT OF THE MYCELIAL PHASE OF *P. brasiliensis* AND THE ANTIGEN E_2 PURIFIED BY IMMUNO-ADSORPTION (RESULTS EXPRESSED IN NUMBER OF PRECIPITATION LINES)

	Crude antigen		*E_2 antigen*
Sera	*DD*	*IEP*	*DD*
Paracoccidioido-mycosis			
01	3	8	1
02	4	9	1
03	7	13	1
04	5	9	1
05	3	5	1
06	0	0	0
07	1	1	1
08	3	6	1
09	5	10	1
10	7	11	1
11	6	9	1
12	0	0	0
13	3	5	1
14	1	2	1
Histoplasmosis			
01	1	2	0
02	3	3	0

DD = Double diffusion test.
IEP = Immunoelectrophoresis.

DISCUSSION

We believe that the antigen E_2 isolated in the course of this work is the antigenic component of band E, the precipitant system characteristic of progressive forms of paracoccidioidomycosis. Three categories of arguments support this assumption. First, the immunological identity reaction yielded by the rabbit monospecific anti-serum anti-E_2 and the serum from the *P. brasiliensis*-infected patient which formed only band E in immunoelectrophoresis. Secondly the high serological reactivity of purified E_2 antigen, and its revelation by the human serum that formed only band E. Finally, the form and position of the precipitant system produced by purified E_2 antigen and the corresponding antibody in immunoelectrophoresis tests.

These results argue against the hypothesis that proposed the cationic protein with alkaline phosphatase activity as the antigen involved in band E formation (8). This hypothesis, which could not be evaluated in the previous work because of the reduced amount of purified antigen produced, must now be rejected. Within the limits of our experience, the band formed by the antigen E_1, carrier of the alkaline phosphatase, remains specific for *P. brasiliensis* extracts. Antigen E_2 was purified by



means of the immunoadsorption procedure described in our previous work (9). The fixation of immunoglobulins of a rabbit monospecific antiserum anti-E_2 over a solid support media of Sepharose, permitted us to retain the substance from a crude metabolic extract of *P. brasiliensis* mycelial growth phase. After elution at acid pH, the final product demonstrated only one immunologically identifiable substance. This substance manifested a neutral or weakly positive charge during gel electrophoresis at pH 8·2, and did not cross-react with rabbit antisera prepared against crude soluble extracts of *B. dermatitidis* and *H. capsulatum*.

The reactivity and specificity exhibited by the purified antigen in the preliminary evaluation here reported, are encouraging. As can be seen from the results of double diffusion tests, all the sera from *P. brasiliensis* infected patients that were found positive in immunoprecipitation reactions contained antibodies that reacted with antigen E_2. Furthermore, the false positive reactions yielded by the two sera from *H. capsulatum*-infected patients and crude extracts of *P. brasiliensis*, disappeared when the purified antigen was used.

These findings make possible the use of E_2 antigen for diagnostic purposes. However, since the purification of the substance involves complex and expensive procedures we think that its reactivity must be evaluated in highly sensitive techniques requiring minimal amounts of antigens, such as the enzyme-linked immunosorbent assay (3) successfully adapted by Farag, Bout and Capron (4) and Bout, Dugimont, Farag and Capron (2) to other purified antigens.

Résumé

L'utilisation de techniques d'immunoadsorption a permis aux auteurs l'obtention pure d'un antigène dénommé E_2, spécifique de *Paracoccidioides brasiliensis*, synthétisé par la phase mycélienne du champignon. Cet antigène qui ne manifeste pas d'activité enzymatique, est un composant du système précipitant E décrit en immunoélectrophorèse. Tous les sérums de patients positifs en immunoprécipitation vis-à-vis d'un extrait métabolique standard de *P. brasiliensis* ont produit un arc de précipitation avec l'antigène E_2 purifié.

Acknowledgements

We are grateful to Drs. Maria B. de Albornoz and Angela Restrepo who sent us the human sera used in this study. We also acknowledge with thanks the skillful technical assistance of Mrs. Réjane Popeye and Mr. Didier Deslee. This work was supported by the "Institut National de la Santé et de la Recherche Médicale" (INSERM) and the "Centre National de la Recherche Scientifique" (CNRS) (ERA 422).

References

1. Axen, R., Porath, H. & Ernback, S. (1967). Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides. *Nature* (Lond.), **214,** 1302–1304.
2. Bout, D., Dugimont, J. C., Farag, H. & Capron, A. Immunodiagnosis of human parasitic diseases by the Enzyme Linked Immunosorbent Assay. In Immunoenzymatic Techniques (INSERM), vol. 2, ed. G. Feldmann, A.S.P., Biological and Medical Press B.V., Amsterdam, March 1976.
3. Engvall, E. & Perlmann, P. (1972). Enzyme Linked Immunosorbent Assay (E.L.I.S.A.) III—Quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen coated tubes. *Journal of Immunology*, **109,** 129–135.
4. Farag, H., Bout, D. & Capron, A. (1975). Specific immunodiagnosis of human hydatidosis by the Enzyme Linked Immunosorbent Assay (E.L.I.S.A.). *Biomédecine*, **23,** 276–278.



5. URIEL, J. (1963). Characterization of enzymes in specific immunoprecipitates. *Annals of the New-York Academy of Sciences*, **103,** 956–979.
6. VAITUKAITIS, J., ROBBINS, J. B., NIESCHLAG, E. & ROSS, G. T. (1971). A method for producing specific antisera with small doses of immunogen. *Journal of Clinical Endocrinology*, **33,** 988–991.,
7. YARZABAL, L. A. (1971). Anticuerpos precipitantes especificos de la blastomicosis sudamericana, revelados por immunoelectroforesis. *Revista do Instituto de Medicina Tropical de Sao Paulo*, **13,** 320–327.
8. YARZABAL, L. A., BIGUET, J., VAUCELLE, T., ANDRIEU, S., TORRES, J. M. & DA LUZ, S. (1973), Analisis immunoquimico de extractos solubles de Paracoccidioides brasiliensis. *Sabouraudia*, **11.** 80–88.
9. YARZABAL, L. A., ANDRIEU, S., BOUT, D. & NAQUIRA, F. (1976). Isolation of a specific antigen with alkaline phosphatase activity from soluble extracts of *Paracoccidioides brasiliensis*. *Sabouraudia*, **14,** 275–280.

